

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
8 August 2002 (08.08.2002)

PCT

(10) International Publication Number
WO 02/060276 A1

(51) International Patent Classification⁷: **A23L 1/03**,
A61K 35/74

LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM,
PH, PL, PT, RO, RU, SD, SE, SG, SI, SK (utility model),
SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU,
ZA, ZM, ZW.

(21) International Application Number: PCT/FI02/00035

(22) International Filing Date: 17 January 2002 (17.01.2002)

(84) Designated States (*regional*): ARIPO patent (GH, GM,
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW),
Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),
European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR,
GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent
(BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR,
NE, SN, TD, TG).

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
20010157 25 January 2001 (25.01.2001) FI

Declarations under Rule 4.17:

— as to applicant's entitlement to apply for and be granted
a patent (Rule 4.17(ii)) for the following designations AE,
AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA,
CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES,
FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE,
KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG,
MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU,
SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG,
UZ, VN, YU, ZA, ZM, ZW, ARIPO patent (GH, GM, KE, LS,
MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent
(AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent
(AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU,
MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI,
CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG)

— of inventorship (Rule 4.17(iv)) for US only

(71) Applicant (*for all designated States except US*): VALIO
LTD [FI/FI]; Meijeritie 6, FIN-00370 Helsinki (FI).

(72) Inventors; and

(75) Inventors/Applicants (*for US only*): MÄYRÄ-MÄKI-
NEN, Annika [FI/FI]; Maurinkatu 4 B 19, FIN-00170
Helsinki (FI). SUOMALAINEN, Tarja [FI/FI];
Valkealantie 10 A 1, FIN-00950 Helsinki (FI). VAAR-
ALA, Outi [FI/FI]; Fredrikinkatu 75 A 5, FIN-00100
Helsinki (FI).

(74) Agent: KOLSTER OY AB; Iso Roobertinkatu 23, P.O.
Box 148, FIN-00121 Helsinki (FI).

Published:

— with international search report

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(81) Designated States (*national*): AE, AG, AL, AM, AT (utility model), AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ (utility model), DE (utility model), DK (utility model), DM, DZ, EC, EE (utility model), ES, FI (utility model), GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU,

(54) Title: COMBINATION OF PROBIOTICS

(57) Abstract: The invention relates to a probiotic combination comprising different combinations of lactobacilli, propionic acid bacteria and/or bifidobacteria. The probiotics are preferably combined with a suitable prebiotic to form a synbiotic. The combination of the invention can be consumed as such or combined with a suitable foodstuff or pharmaceutical product, and it is therapeutically useful for example for stimulating the immune system and for general health improvement.

WO 02/060276 A1

COMBINATION OF PROBIOTICS

FIELD OF THE INVENTION

[0001] The invention relates to a combination of probiotics, the combination comprising lactobacilli, propionic acid bacteria and/or bifidobacteria in various combinations. The probiotics are preferably combined with a suitable prebiotic to produce a synbiotic. The combination of the invention may be consumed as such or combined with a suitable foodstuff, such as a dairy product or a drink, and it is therapeutically useful for example for stimulating the immune system and for general health improvement.

10 BACKGROUND OF THE INVENTION

[0002] Probiotics are live microbes which, when administered to man or animals, promote the well being of the host by improving the intestinal microbial balance (Fuller, R. Probiotics in man and animals, 1989, J. Appl. Microbiol. 66:365-378). The best-documented probiotics include *L. rhamnosus* LGG, *L. johnsonii* LAI, *L. casei* Shirota and *Bifidobacterium lactis* Bb12. In addition, a number of other probiotics have been described in the literature of the art (see for example M. E. Sanders & J. H. in't Veld 1999. Antonie van Leeuwenhoek 76:293-315, Kluwer Academic Publishers). The health-promoting effects of probiotics include the balancing and maintenance of intestinal flora, stimulation of the immune system and anti-carcinogenic activity. The useful effects of probiotics in human intestines are based on several factors caused by live bacterial cells, their cell structures and metabolic products. Probiotics are usually administered in nutrients or as capsules.

[0003] A bacterium may be referred to as a probiotic if it essentially meets the following requirements (Lee, Y-K and Salminen, S. 1995 The coming age of probiotics. Trend Food Sci Technol, 6:241-245): it remains viable in the demanding conditions prevailing in the digestive tract (low pH of the stomach, acids of the digestive system, etc.); attaches to the walls of the intestine; metabolizes in the intestine; is technologically applicable (endures processing); exhibits clinically tested and reported health effects; and is safe to consume.

[0004] Prebiotics are nondigestible food ingredients which promote the health of humans by selectively stimulating the growth and activity of one or some probiotic bacteria in the colon (Gibson, G.R. and Roberfroid, M.B. 1995. Dietary modulation of the human colonic microbiota - introducing a concept of prebiotics. J. Nutr. 125:1401-1412). A prebiotic is usually a

nondigestible carbohydrate (oligo- or polysaccharide) or a sugar alcohol which is not degraded or absorbed in the upper digestive tract. Known prebiotics used in commercial products include inulin (fructo-oligosaccharide, or FOS) and transgalacto-oligosaccharides (GOS or TOS).

5 **[0005]** A synbiotic is defined as a combination of a prebiotic and a probiotic, the prebiotic promoting the viability of the added microbe and its attachment to the intestine, thereby promoting health (Gibson and Roberfroid 1995, supra). When nondigestible carbohydrates that have passed through the small intestine are fermented in the colon, short-chain fatty acids, other
10 organic acids, alcohols, hydrogen and carbon dioxide, for example, are formed (Gibson and Roberfroid 1995, supra). The primary fatty acids produced in fermentation are acetic acid, butyric acid and propionic acid (Cummings, J.H. Short-chain fatty acids, in: Human Colonic Bacteria: Role in Nutrition, Physiology and Pathology, G.R. Gibson and G.T. Macfarlane (eds.), pp. 101 -
15 130, CRC Press, Boca Raton, 1995). An increase in the number of short-chain fatty acids would be generally advantageous. Nondigestible carbohydrates are the principal substrate for colonic microbes, although they also may include compounds the intestinal fermentation of which is disadvantageous (Gibson and Roberfroid, 1995, supra).

20 **[0006]** Human digestive tract accommodates a plural number of bacteria which live in symbiosis with the host. There are great differences in microbial content between the different parts of the tract, about 95% of all the intestinal bacteria appearing in the colon, which is the most important part of the intestines. Over 400 bacterial species have been estimated to thrive in the
25 colon. In addition to these, the intestines contain microbes known as transient microbes (G.R. Gibson and M.B. Roberfroid (eds.) Colonic Microbiota; Nutrition and Health. Kluwer Academic Publisher, Dordrecht, 1999). The dominating species are the following: *Bacteroides*, *Bifidobacterium*, *Coprococcus*, *Peptostreptococcus*, *Eubacterium* and *Ruminococcus*. The
30 number of species *Lactobacillus*, *Streptococcus*, *Fusobacterium*, *Veillonella*, *Propionibacterium* and *Enterobacteriaceae* is slightly less. Some of the species represent useful microbes, whereas others may even be harmful. The average microbial content in feces is 10^{12} cfu/g (per dry matter). Bacteria degrade and ferment those food ingredients in the colon, which are not absorbed in the small
35 intestine, the end products of the fermentation being absorbed in the intestine for use by the body. In addition to nutrition, the microbial balance of the colon is

of major significance to the state of health of a man (Tannock, G.W. 1998. Studies of the intestinal microflora: A prerequisite for the development of probiotics, Int. Dairy J. 8:527 - 533). Changes in the composition of the intestinal flora or a sudden reduction in the amount of it (due to severe diarrhea, antibiotics treatment, etc.) increase the infectivity of potentially pathogenic species, which may have serious consequences (outbreak of allergies, intestinal diseases, cancer).

[0007] The β -glucuronidase enzymes produced by intestinal bacteria are assumed to contribute to the formation of carcinogenic compounds, for example. Steroids and other carcinogenic compounds metabolize in the liver and then conjugate with glucuronic acid. The bile delivers the conjugated glucurone compound to the small intestine and from there the compound passes further to the colon where the glucuronidase enzymes can hydrolyse the compound, thereby releasing toxic compounds into the colon (Rowland, I.R. 1995. Toxicology of the colon: role of the intestinal microflora, in: Human Colonic Bacteria, Role in nutrition, physiology, and pathology. Editors: Gibson, G.R. and Macfarlane, G.T., pp. 155-174, Boca Raton: CRC Press). It is assumed that species of *Eubacterium*, *Bacteroides* and *Clostridium* release greater amounts of these harmful enzymes into the intestines than representatives of species of *Bifidobacterium* and *Lactobacillus*. This would thus provide one reason why it would be advantageous that the intestinal flora is composed of bifidobacteria and lactobacilli.

[0008] In addition, glycosides originating from vegetables and tea, for example, are not absorbed in the small intestine and pass to the colon, where they may be hydrolysed by the action of β -glucosidases to form toxic or mutagenic aglycone compounds (Goldin, B.R. 1990. Intestinal Microflora: metabolism of drugs and carcinogens. Annals of Medicine 22:43-48).

[0009] Moreover, the intestinal flora produces urease enzyme which degrades urea into ammonia. High amounts of ammonia may be toxic to the epithel cells of the intestine (Moblely, H.L.T. and Hausinger, R.P. 1989. Microbial ureases: significance, regulation and molecular characterization. Microbiological Reviews 53:85-108).

[0010] Human intestinal flora is formed during the early years of life and no major changes take place in its composition thereafter. Only minor changes within the species may take place (in bifidobacteria, for example).

[0011] Along with the increased understanding of the importance of intestinal flora, research has thus been actively focused on discovering the factors that can be used to influence the composition of the flora and its operation (viability) in such a way that beneficial bacterial species would be strengthened and harmful ones reduced. It is assumed that the operation of microbes can be influenced by prebiotics that promote beneficial bacteria. Extensive research has been carried out into galactooligosaccharides (GOS) which are di-, tri-, tetra-, penta- and hexasaccharides and which primarily contain galactose units. They are prepared enzymatically from lactose and the content of the end product depends on the enzyme used (Matsumoto, K. et al. 1993. Galactooligosaccharides, in: Oligosaccharides. Production, properties and applications. Ed. Nakakuki, T., Japanese Technology Reviews. Vol. 3. No. 2., pp. 90-116, Gordon and Breach Science Publishers, Switzerland, Australia). GOS has earlier been demonstrated to exhibit for example bifidogenic properties, i.e. those promoting the growth of bifidobacteria (Ito, M. et al. 1990. Effect of administration of galactooligosaccharides on the human faecal microflora, stool weight, and abdominal sensation. Microb. Ecol Health Dis. 3:285-292).

DESCRIPTION OF THE BACKGROUND ART

[0012] Both products containing an individual probiotic strain and combinations of a plural number of different probiotics have been abundantly described in the literature of the art. Synbiotics have also been described in the literature of the art.

[0013] Publication EP 904 784, N. V. Nutricia, for example, describes a probiotic product containing *Bifidobacterium*, *Enterococcus faecium* and *Lactobacillus*. In addition, the product may contain prebiotics, such as polysaccharide or non-degradable starch, and immunoglobulines, vitamins, etc. According to the publication, the product has a health-promoting effect in that it stimulates the immune system, for example. The effect has not, however, been shown in clinical tests, neither has any other biological activity been studied.

[0014] WO 00/33854, N. V. Nutricia, describes a product comprising a probiotic and oligosaccharides. The probiotics particularly referred to are *Lactobacillus* and *Bifidobacterium*, although *Pediococcus*, *Propionibacterium*, *Leuconostoc* and *Saccharomyces* are also mentioned. The

prebiotics involved include transgalactooligosaccharides (TOS) and fructooligosaccharides (FOS). According to the publication, a particularly advantageous combination comprises *Lactobacillus rhamnosus* and a transgalactooligosaccharide or a hydrolysis product of potato galactane, this combination and its preparation being also illustrated in the examples. According to the publication, the product has health-promoting effects and it is particularly useful in the treatment of intestinal disorders. However, its activity has not been demonstrated in any way.

[0015] WO 97/34615, University of New South Wales, describes a probiotic composition containing, in addition to one or more probiotics, resistant (non-degradable) starch and oligosaccharide, a synergistic effect being obtained between the three components. The probiotics mentioned are *Saccharomyces*, *Bifidobacterium*, *Bacteroides*, *Clostridium*, *Fusobacterium*, *Propionibacterium*, *Lactococcus*, *Streptococcus*, *Enterococcus*, *Staphylococcus*, *Peptostreptococcus* and *Lactobacillus*, and the oligosaccharides include, among many others, fructo- and galacto-oligosaccharides. The examples show the synergistic effect of bifidobacteria, maize starch and fructooligosaccharides on the amount of bifidobacteria. Any therapeutic effect, however, has not been described.

[0016] US 5,895,648, Sitia-Yomo S.p.A., describes a probiotic composition comprising, in the form of lyophilized, live bacteria, at least two species of bifidobacteria and at least two species of lactobacilli or streptococcus combined with one or more oligosaccharides. The composition comprises altogether 4 to 20 parts by weight of probiotics and 5 to 22 parts by weight of oligosaccharides, of which for example galacto- and fructooligosaccharides, inuline in particular, are mentioned. The probiotics mentioned include *Bifidobacterium breve*, *Bifidobacterium infantis*, *Bifidobacterium longum*, *Bifidobacterium bifidum*, *Lactobacillus acidophilus*, *Lactobacillus bulgaricus*, *Lactobacillus casei*, *Lactobacillus plantarum*, *Streptococcus thermophilus* and *Streptococcus faecium*. According to the publication, a mixture containing probiotics and prebiotics can be added to milk-based desserts, milks or juices to balance the functioning of the intestine. Any biological activity of the combination has not, however, been described.

[0017] The publication *Milchwissenschaft* (1988) Vol. 53, No. 11, pp. 603-605, describes PAB-milk which comprises three bacterial strains: *Propionibacterium freudenreichii* subsp. *shermanii* MTCC 1371, *Lactobacillus*

acidophilus R. and *Bifidobacterium bifidum* NDRI. Prebiotics have not been added to the product. According to the publication, PAB-milk is suitable for babies and children, also for those suffering from lactose intolerance.

5 **[0018]** WO 99/10476 describes the stimulating effect of specific bacterial strains, i.e. *Lactobacillus rhamnosus* HN001 (NM97/09514) and HN067 (NM97/01925), and *Lactobacillus acidophilus* HN017 (NM97/09515) and *Bifidobacterium lactis* HN019 (NM97/09513), on the immune system, measured as enhanced phagocytosis. The strains can be used individually, or added to dairy products or pharmaceutical preparations.

10 **[0019]** US 5,902,578, Abbott Laboratories, relates to a method for preventing and treating diarrhea with a mixture of *Lactobacillus reuteri*, *Lactobacillus acidophilus* and *Bifidobacterium infantis*, the mixture being prepared as a powder, liquid or pills.

15 **[0020]** Biological and therapeutic effects of probiotics and synbiotics of the above type have also been described in the background art. As examples may be mentioned Gallagher, D. et al. Journal of Nutrition (1996) Vol. 126, No. 5, pp. 1362-1371, which describes the effects of bifidobacteria and *Lactobacillus acidophilus* on colon cancer in rats and states that the best results were obtained by using both the bacteria and a fructooligosaccharide, and Kirjavainen, P. et al. Clinical and Diagnostic Laboratory Immunology (Nov 20 1999) Vol. 6, No. 6, pp. 799-802, which describes the positive effects of two, separately studied, lactic acid strains, *Lactobacillus rhamnosus* GG and *Propionibacterium freudenreichii* subsp. *shermanii* JS, on the lymphocyte levels, and thereby the immune response, of mice. The combination of these 25 two bacteria has not been described or studied.

30 **[0021]** The combining of probiotics with other substances having therapeutic effects has also been described in the literature of the art. For example, WO 97/29762 and WO 97/29763, Procter & Gamble Company, describes the use of lactobacilli and bifidobacteria combined with galacto- or fructooligosaccharides together with a plant of the genus *Ericaceae*, or an extract thereof for the treatment of urinary tract infections and intestinal disorders, and WO 00/29007, Reddy, describes the combining of probiotics, such as *Lactococcus*, *Lactobacillus*, *Pediococcus*, *Streptococcus*, *Propionibacterium*, *Brevibacterium*, *Penicillium* and *Saccharomyces* with herb- 35 based natural products and drug-like substances.

[0022] Although probiotics and synbiotics have been extensively studied, good and versatile commercial products are not available to any significant extent. Consequently, there is continued, evident need to offer the consumers new products having clearly demonstrated probiotic effects and produced in a form that allows them to be used as a convenient part or supplement, for example, of the every-day diet.

BRIEF DESCRIPTION OF THE INVENTION

[0023] It is therefore an object of the present invention to provide a new probiotic-containing product the probiotic effect of which has been clearly demonstrated, which is pleasant to use, and healthy for the consumer.

[0024] These objects were achieved with a new combination of the invention which comprises a plural number of probiotics. The present invention is thus based on a new combination comprising (2) lactobacilli strains, a propionic acid bacterium and/or a bifidobacterium. In addition, the combination preferably comprises a prebiotic supporting the growth of the above-mentioned microbes.

[0025] According to the invention, two strains of lactobacilli are mainly used, i.e. *Lactobacillus rhamnosus* LGG (ATCC 53103) and *Lactobacillus rhamnosus* LC705 (DSM 7061). The propionic acid bacterium is usually *Propionibacterium freudenreichii* ssp. *shermanii* PJS (DSM 7067). The bifidobacterium may be any bifidobacterium having a probiotic effect, typically strains belonging to the species *Bifidobacterium breve*, *Bifidobacterium infantis*, *Bifidobacterium longum*, *Bifidobacterium bifidum* and *Bifidobacterium adolescentis* are used.

[0026] At least three of these bacteria are usually included, and the combination preferably includes *Lactobacillus rhamnosus* LGG and/or *Propionibacterium freudenreichii* ssp. *shermanii* PJS.

[0027] The most preferred combination is a mixture of four strains, *Lactobacillus rhamnosus* LGG (ATCC 53103), *Lactobacillus rhamnosus* LC705 (DSM 7061), *Propionibacterium freudenreichii* ssp. *shermanii* PJS (DSM 7067) and *Bifidobacterium infantis* Bbi99 (DSM 13692). However, any bifidobacterium (such as Bbl2) may be included in the combination, if desired. The prebiotic used is preferably galactooligosaccharide (GOS).

[0028] Another preferred composition is a combination of a bifidobacterium and a propionic acid bacterium, in which any bifidobacterium

can be used together with *Propionibacterium freudenreichii* ssp. *shermanii* PJS (DSM 7067).

[0029] The new combination can be used as such or as a part of another product, such as a pharmaceutical or a food product. The combination of the invention has an advantageous effect on the human intestinal balance in that it increases the entero-lactone production and reduces a disadvantageously high pH value. The combination also influences the immune response by increasing the amount of lymphocytes and that of γ -interferon (IFN) and by reducing the formation of carcinogenic substances. The combination of the invention is thus useful for the prevention and treatment of intestinal disorders, allergies and cancer and for promoting general health.

[0030] In accordance with the present invention, the combination is thus also applicable as a therapeutic substance and in the preparation of therapeutic substances.

15 DETAILED DESCRIPTION OF THE INVENTION

[0031] Of the strains used in the invention, *Lactobacillus rhamnosus* GG (LGG), *Lactobacillus casei* ssp. *rhamnosus* LC705 and *Propionibacterium freudenreichii* ssp. *shermanii* JS (PJS) have been described in the prior art. *Bifidobacterium infantis* Bbi99, which may be included in the combination, is a new strain and will be described in greater detail below.

[0032] *Lactobacillus rhamnosus* GG (LGG) is described for example in US Patent 5,032,399, Gorbach & Goldin. The strain is isolated from human feces, it is able to grow well in pH 3 and survives even lower pH values as well as high bile acid contents. The strain exhibits excellent adhesion to both mucus and epithelial cells. Lactic acid yield from glucose is good: when grown in MRS broth, the strain produces 1.5 - 2% of lactic acid. The strain does not ferment lactose and thus it does not produce lactic acid from lactose. The strain employs the following carbohydrates: D-arabinose, ribose, galactose, D-glucose, D-fructose, D-mannose, rhamnose, dulcitol, inositol, mannitol, sorbitol, N-acetylglucosamine, amygdalin, arbutin, esculin, salicin, cellobiose, maltose, saccharose, trehalose, melezitose, gentibiose, D-tagatose, L-fucose, and gluconate. The strain grows well at +15 - 45°C, the optimum temperature being 30 - 37°C. *Lactobacillus rhamnosus* GG is deposited with the depository authority American Type Culture Collection under accession number ATCC 53103.

[0033] *Lactobacillus casei* ssp. *rharnosus* LC705 is described in greater detail in FI Patent 92498, Valio Oy. LC705 is a gram-positive short rod occurring in chains; it is homofermentative; weakly proteolytic; grows well at +15 - 45°C; does not produce ammonia from arginine; is catalase-negative; when
5 grown in MRS broth (LAB M), the strain produces 1.6% lactic acid having an optical activity of the L(+) configuration; the strain decomposes citrate (0.169%), thereby producing diacetyl and acetoin; the strain ferments at least the following carbohydrates (sugars, sugar alcohols): ribose, galactose, D-glucose, D-fructose, D-mannose, L-sorbose, rhamnose, mannitol, sorbitol,
10 methyl-D-glucoside, N-acetylglucosamine, amygdalin, arbutin, esculin, salicin, cellobiose, maltose, lactose, sucrose, trehalose, melezitose, gentiobiose, D-turanose and D-tagatose. LC705 adheres weakly to mucus cells, but moderately to epithelial cells. The viability of the strain is good in low pH values and high bile acid contents. The strain survives well a salinity of 5% and fairly
15 well a salinity of 10%. *Lactobacillus casei* ssp. *rharnosus* LC705 is deposited with the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) under accession number DSM 7061.

[0034] *Propionibacterium freudenreichii* ssp. *shermanii* JS (PJS) is also described in greater detail in FI Patent 92498, Valio Oy. PJS is a gram-
20 positive short rod; it ferments glucose, fructose, galactose and lactose; it ferments well lactate; and its optimum growth temperature is 32°C. The viability of the strain in low pH values and high bile acid contents is excellent. *Propionibacterium freudenreichii* ssp. *shermanii* JS is deposited with the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ)
25 under accession number DSM 7067.

[0035] *Bifidobacterium infantis* Bbi99 has been isolated from the feces of a healthy newborn. *B. infantis* Bbi99 is a gram-positive pleomorphic rod. The strain is catalase-negative, fructose-6-phosphate-phosphoketolase-positive

Zellkulturen GmbH (DSM), in accordance with the Budapest Treaty, under accession number DSM 13692 on 28 August 2000.

5 **[0036]** The combination of the invention may also include other microorganisms, such as microorganisms and probiotics contained in starters used in the dairy industry. There are numeral well-documented strains of starters, which are commercially available from producers such as Hansen A/S, Denmark, and Danisco/Wiesby GmbH, Germany.

10 **[0037]** To prepare the combinations of the invention, the microorganisms are cultivated using processes conventional in the art. They can be cultivated as pure cultures or as different mixed cultures. The cultures can be used as such or they may be processed as desired for example by purifying, concentrating, lyophilising or finishing them to produce various preparations.

15 **[0038]** A sufficient amount of probiotics is used in the combination to produce the desired probiotic effect. The amount of each probiotic may thus vary within a broad range, depending on, for example, the strains used and their number, the total amount of cells of the probiotics, the total daily dose, and on other properties and ingredients of the product. A daily dose of the combination usually comprises about 10^6 - 10^{10} cfu of probiotics.

20 **[0039]** One or more prebiotics are preferably added to the combination to form a synbiotic. The prebiotic is selected according to the microorganisms included in the combination concerned such that it will support the growth of the microorganisms. Suitable prebiotics may include e.g. oligosaccharides, particularly galactooligosaccharide (GOS),
25 palatinoseoligosaccharide, soybean oligosaccharide, gentiooligosaccharide, xylooligomers, non-degradable starch, lactosaccharose, lactulose, lactitol, maltitol, polydextrose, or the like. A sufficient amount of the prebiotic is added to the synbiotic to produce a prebiotic effect. What is a sufficient amount is determined for example according to the strain concerned, the amount of the
30 prebiotics included and the other contents and the application of the product. The amount therefore also varies within a wide range; it may be from 0.5 to 5 g in a daily dose, for example.

35 **[0040]** The prebiotic does not necessarily need to be included in the combination. Depending on the end product and the purpose of use, it may be better to consume the prebiotic separately, although approximately at the same time with the probiotic combination. In some cases it may suffice to only

consume the probiotic combination, the prebiotic being thus not needed at all. An example of this is a case where the intestinal conditions of the host are suitable for the growth of probiotics without an added prebiotic being needed, and when the prebiotic is contained in the normal diet (if it is consumed in porridge or rye bread, for example).

[0041] The present invention has shown that the microorganisms used meet the criteria set for probiotics: they survive well in the demanding conditions of the digestive tract, adhere well to intestinal cells and multiply well in the intestines. They have also been shown to exhibit excellent biological effects; for example, they increase the number of microorganisms desirable from the point of view of health and reduce the number of harmful microorganisms in the intestine, they reduce the activity of harmful enzymes and thereby the formation of harmful, or even carcinogenic, substances, and they have a stimulating effect on the immune response.

[0042] The combination of the invention can be used as such or in the form of capsules, pills or tablets, for example, manufactured in conventional processes of preparing pharmaceutical products. The combination of the invention may also be added to diverse edible products, such as foodstuffs, products of the beverage or confectionery industry, health-promoting products, natural products, etc. In the context of the present invention, products containing the combination of the invention, such as dairy products, particularly yogurts and other fermented milk products; cheeses and spreads; children's food; juices and soups; and capsules are preferred. A product in the form of a capsule usually only contains the probiotic combination, the prebiotic being consumed separately.

[0043] The end products are prepared in conventional processes, the combination being added either in connection with the preparation or thereafter, during the finishing of the end product.

[0044] The invention is described in greater detail with reference to the following examples, which are only intended to illustrate the invention and not to restrict its scope in any way.

Example 1

Preparation of the combination

[0045] The combination was prepared from a bacterial mixture, adding, when desired, galacto-oligosaccharide (GOS) as a prebiotic. The bacterial mixture was formed from bacterial cultures (concentrates or freeze-

dried powders) of four strains, i.e. *Lactobacillus rhamnosus* LGG (ATCC 53103), *Lactobacillus rhamnosus* LC705 (DSM 7061), *Propionibacterium freudenreichii* ssp. *shermanii* PJS (DSM 7067) and *Bifidobacterium infantis* Bbi99 (DSM 13692).

5 **[0046]** Both LGG and bifidobacterium were cultivated individually.

[0047] LGG was cultivated in a medium comprising 5.0% of whey permeate (Valio Oy), 0.5% of casein hydrolysate (Valio Oy), 0.5% of technical yeast, and 0.0015% of $\text{MnSO}_4 \times \text{H}_2\text{O}$. The components of the medium were dissolved into water and the medium was sterilized (for 20 min at 120°C). The cultivation was carried out at a temperature of 37°C and a pH of 5.8 (adjusted with NH_4OH) for about 18h and at a mixing rate of 100 rpm. After the cultivation the bacterial cells were concentrated, washed and freeze-dried using a 10% (v/v) protective agent supplement, such as a saccharose broth of 46%, or a similar alternative known to those skilled in the art. The final bacterial content was $>1 \times 10^9$ cfu/ml in the cultivation, $>1 \times 10^{10}$ cfu/g in the concentrate and $>1 \times 10^{11}$ cfu/g in the freeze-dried powder.

[0048] The composition of the growth medium of bifidobacteria was the following: whey permeate 4% (Valio Ltd.), casein hydrolysate 1.0% (Valio Ltd.), technical yeast extract 1.0% (LAB M), cystein-HCl 0.03% (Merck, Darmstadt, Germany). Other ingredients of the medium were first dissolved into water, then the cystein-HCl was added and the medium was sterilized (for 20 min at 120°C). The cultivation was carried out in about 18-20 hours at a temperature of 37°C and a pH of 6.7 (adjusted with NH_4OH) and at a mixing rate of 100 rpm. The bacterial content of the cultivation was $>1 \times 10^9$ cfu/ml. After the cultivation the bacteria cells were concentrated, washed and freeze-dried using a 10% (v/v) protective agent supplement, such as a saccharose broth of 46%, or a similar alternative known to those skilled in the art. The bacterial content in the concentrate was $>1 \times 10^{10}$ cfu/g and in the freeze-dried powder $>1 \times 10^{11}$ cfu/g.

[0049] LC705 and PJS were cultivated together by inoculating bacterial cells to a whey-based growth broth in a ratio of 1:2. The whey-based growth medium contained 3.5 - 5% of whey permeate (Valio Oy), 1.0% of caseinhydrolysate and 1.0% of yeast extract (Valio Oy). The strains were cultivated for three days at 30°C with the pH maintained at 4.5 by means of an automated pH adjustment. After the cultivation, the content of each bacterial strain was $>1 \times 10^9$ cfu/ml. After the cultivation the bacterial cells were concentrated, washed and freeze-dried using a 10% (v/v) protective agent

supplement, such as a saccharose broth of 46% or a similar alternative known to a person skilled in the art. The content of each of the bacteria in the concentrate was $>1 \times 10^{10}$ cfu/g and in the freeze-dried powder $> 1 \times 10$ cfu/g.

[0050] The strains may also be cultivated separately. In that case
 5 LC 705 is cultivated as described above in connection with the joint cultivation, only the cultivation time is 1 day at 30°C. PJS is cultivated in a whey-based growth medium comprising 2% of whey permentate, 1.0% of casein-hydrolysate and 1% of yeast extract. The strain is cultivated for 3 days at 30°C in a pH value of 6.3, after which the process is carried out as above.

10 [0051] The concentrates or the powders are mixed in a ratio of 1:1:1. When LC705 and PJS have been cultivated separately, the mixing ratio is 1:1:1:1. The obtained mixture of concentrates or freeze-dried powders is used as a probiotic portion in the different product applications of the combination. The mixture is added to the product application to obtain the
 15 following final bacteria content in the product:

	LGG	$>10^6$ cfu/g of the product
	LC705	$>10^6$ cfu/g of the product
	PJS	$>10^6$ cfu/g of the product
20	Bifidobacterium	$>10^6$ cfu/g of the product

[0052] GOS (Valio Oy) was added, when desired, as a separate product to the product application to obtain a GOS concentration of about 0.5 - 5 g/dose in the product.

25 Example 2

Adhesive properties of the strains and their tolerance in intestinal conditions

[0053] Adhesion of the probiotic strains to mucus was tested in accordance with Ouweland et al. (Ouwehand, A. C., Kirjavainen, P. V.,
 30 Grönlund, M. - M., Isolauri, E., and Salminen, S. J. 1999. Adhesion of probiotic micro-organisms to intestinal mucus. Int. Dairy J. 9:623-630). LGG and probiotic bacterium PJS adhered excellently to the intestinal mucus, Bbi99 moderately and LC705 weakly. Adhesion is a prerequisite for the microbe to produce beneficial effects in the intestines. On the other hand, LC705 is known to adhere
 35 well to epithel cells, similarly as LGG (Lehto, E. and Salminen S. 1997. Adhesion of two *Lactobacillus* strains, one *Lactococcus* strain and one

propionibacterium strain to cultured human intestinal CaCO-2 cell lines. Bioscience Microflora 16: 13 - 17). This property is beneficial when there is an imbalance in the intestine and the protective mucus has been weakened.

5

Table 1. Adhesive properties of probiotic strains

Strain	Adhesion (+Std.)	%
LGG	26.3 ± 1.3	
LC705	0.7 ± 0.2	
PJS	24.9 ± 2.2	
Bbi99	4.6 ± 2.3	

[0054] *In vitro* tests have shown the strains to endure physiological bile salt contents and the low pH of the stomach.

- [0055]** The strains were tested at different pH values in MRS broths the pH of which had been adjusted with lactic acid to values pH 4, pH 3 and pH 2. The strain to be studied (fresh culture) was inoculated into a pH broth of 1% and grown at 37°C for 3 hours, after which the content of live cells was determined using an agar suitable for the strain (see Table 5). The strains maintained their viability in the 3-hour process in a pH value of 3 excellently.
- The propionic bacterium remained viable even in pH 2. In the test the bacteria were not protected by components carried by food (such as fat) and therefore they may be assumed to preserve even better when consumed *in vivo* together with food.

Table 2. Cell contents of strains cultivated in pH-adjusted MRS broths

	Initial content cfu/ml	pH 4 cfu/ml	pH 3 cfu/ml	pH 2 cfu/ml
Bbi99	1 x 10 ⁷	2 x 10 ⁷	<10	<10
PJS	1 x 10 ⁸	9 x 10 ⁷	3 x 10 ⁷	1 x 10 ⁴
LGG	1 x 10 ⁷	2 x 10 ⁷	1 x 10 ⁷	<10 ²
LC705	6 x 10 ⁷	2 x 10 ⁸	4 x 10 ⁷	<10 ²

[0056] With regard to bile salt tolerance, the strains were tested in MRS broths that contained 0.3% and 0.5% of Oxgal (Sigma) bile salt by inoculating 1% of the fresh culture into the bile salt MRS broth to be studied.

The strains were cultivated in the broths for 3 hours at 37°C, after which the content of live cells was determined using an agar suitable for the strain (see Table 5). All strains survived the treatment excellently.

5 **Table 3. Cell contents of strains cultivated in bile salt contents of 0.3 % and 0.5 %**

	Initial content (cfu/ml)	Bile salt 0.3 %	Bile salt 0.5 %
Bbi99	3×10^7	1×10^7	1×10^7
PJS	6×10^7	6×10^7	5×10^7
LGG	1×10^7	2×10^7	9×10^6
LC705	1×10^7	2×10^7	1×10^7

Example 3

Selecting a prebiotic suitable for the combination

- 10 **[0057]** Alternative prebiotics were studied on individual strains by cultivating each strain in a sugar-free MRS broth to which was added 1% of the prebiotic to be studied. Each strain was cultivated for 1-2 days at its optimum temperature. Bacterial growth was observed during the test by determining the turbidity of the cultivation by spectrophotometry. As is shown
- 15 in Table 4, best promoter for growth in all the four strains was a galacto-oligosaccharide (GOS) supplement of 1%.

Table 4. The effect of prebiotics on bacterial growth

Prebiotic	LGG	LC705	PJS	Bbi99
GOS	++	++	++	+
FOS	-	-	-	-
Xylooligomers	+	++	+	+
Polydextrose	++	++	+	-
Arabinooligomers	-	-	-	-
Pectinooligomers	-	-	-	-
Xylitol	-	-	-	-
Maltitol	-	++	-	-
Lactitol	-	++	-	-

Example 4

Preparation of the end product

[0058] A functional drink (in Finnish 'tehojuoma'; Valio Oy) was used as a base for preparing a juice (dosage 65 ml/day) to which was added 0.1 g of freeze-dried mixture of bacteria / dose (= 65 ml) and 3.8 g of 70% GOS syrup / dose (= 65 ml). Corresponding juice with no added GOS syrup or bacterial mixture was used as a control.

[0059] The bacterial contents of the finished juice were as follows:

LGG	> 10 ⁷ cfu/ml
LC705	> 10 ⁷ cfu/ml
PJS	> 10 ⁸ cfu/ml
Bbi99	> 10 ⁷ cfu/ml

[0060] The product was used in the following clinical tests in which pro = juice + probiotic supplement and syn = juice + probiotic + prebiotic supplement.

Example 5

Clinical effects of the combination of the invention

[0061] The drink described in example 4, which contained the above described probiotic combination (Pro) or the probiotic combination and a prebiotic (Syn), was clinically tested on 20 males. The test persons took the drink daily in accordance with the study plan and during the study they were not allowed to consume any other probiotic-containing product. The study scheme was such that the test started with a run-in period continued by a probiotics period of two weeks and a subsequent synbiotics period, and it ended with what is known as a wash-out period.

The study scheme and schedule

week 8	N (week 11)		N (week 13)		N (week 15)		N (week17)
I 3 week	*I	2 week	*I	2 week	*I	2 week	*I
run-in	pro		Syn		normal diet		
without					without		
probiotic					probiotic		

[0062] At the end of each period, the test persons gave a feces sample and a blood sample (= N).

[0063] Microbes and enzymes were analysed from the feces samples and enterolactone content and immune response from the blood.

5.1. Microbes

Amount of microbes

- 5 [0064] The total lactic acid bacterial content, LGG, LC705, the total propionic acid bacterial content, PJS, and the total bifidobacteria content were determined applying methods known in the art and the parameters shown in Table 5.

10 **Table 5. Methods for determining microbes**

Determination	Agar	Cultivation temperature/ time	
Lactobacilli	MRS	37°C/3 days	anaerobic
LGG	MRS + 0.005 % vancomycin (Sigma)	37°C/3 days	
LC705	MRS + 0.005 % Vancomycin	37°C/3 days	
Propionic bacteria	Mod. YEL	30°C/7 days	anaerobic
PJS	Mod. YEL	30°C/7 days	anaerobic
Bifidobacteria	Raffinoseagar (RB)	37°C/2 days	anaerobic

Effect on the intestinal bacterial cell content *in vivo*

- 15 [0065] The contents of LGG, LC705 and PJS increased significantly in the samples of the test persons during the period they used the probiotic-containing product (Table 6). Since bifidobacteria contents were high from the very beginning of the test, changes have presumably only taken place within the species.

Table 6. Bacterial contents (log, cfu/g of feces; \pm std.)

	Initial content cfu/g	After intake of probiotic, cfu/g	After intake of synbiotic, cfu/g	Wash-Out cfu/g
MHB (tot)	6.0 ($\pm 1,2$)	6.1 ($\pm 1,2$)	6.1 ($\pm 1,0$)	4.5 ($\pm 1,6$)
LGG	2.3 ($\pm 1,0$)	4.7 ($\pm 1,7$)	5.3 ($\pm 1,2$)	3.0 ($\pm 1,6$)
LC705	2.0 (± 0)	5.2 ($\pm 1,3$)	5.4 ($\pm 1,0$)	2.8 ($\pm 1,1$)
Propion	2.7 ($\pm 1,4$)	5.7 ($\pm 1,6$)	5.6 ($\pm 1,4$)	2.4 ($\pm 1,7$)
Bifidobacteria	8.2 ($\pm 1,5$)	8.6 ($\pm 2,1$)	8.8 ($\pm 2,2$)	8.3 ($\pm 2,1$)

- 5 **[0066]** Addition of synbiotic to the product consumed in the intake test improved the viability of the added probiotics in the intestine. This is shown by the increase in the LGG content during the synbiotics period, for example.

Effect on pH value

- 10 **[0067]** The pH level of persons having an initial pH level of over 7 decreased in the groups that took the probiotic mixture and the synbiotic, whereas no decrease was observed in persons with an initial pH value lower than 7 (Table 7).

Table 7.

Change in pH after probiotic and synbiotic supplement

	Control	Probiotic alone	Synbiotic
pH>7	7.2	6.9	6.7
pH<7	6.6	6.7	6.6

5.2. Enzymes

- 20 **[0068]** The feces samples were processed as described by Ling et al. (Ling, W-H., Korpela, R., Mykkänen, H., Salminen, S., and Hänninen, O. 1994 Lactobacillus GG supplementation decreases colonic hydrolytic and reductive activities in healthy female adults. Journal of Nutrition 124, 18-24).

- 25 **[0069]** β -glucuronidase and β -glucosidase were determined as described by Freeman (Freeman, H. J. 1986. Effects of differing purified cellulose pectin, and hemicellulose fibre on faecal enzymes in 1,2-dimethyl - hydrazine-induced rat colon carcinogenesis. Cancer Research 46: 5529 - 5532)

and urease according to the instructions of the manufacturer (Boehringer Mannheim cat. no. 542946).

- 5 **[0070]** During the test periods, there was a decrease in the contents of β -glucuronidase, urease and β -glucosidase during the probiotic intake and the synbiotic intake (Table 8). After the intake, the enzyme levels returned to normal. The synbiotic had a stronger decreasing effect on the enzyme levels than the probiotic mixture.

Table 8. Changes in enzyme contents (nmol/min/g of feces)

	Urease		Glucuronidase		Glucosidase	
		Change%		Change%		Change%
Initial level	1080		292		746	
Probiotic	895	-17	214	-27	673	-9,8
Synbiotic	592	-45	186	-36	448	-40
Wash-Out	980		227		640	

10

- 15 **[0071]** Metabolism of glucosidase and glucuronidase produces carcinogenic compounds. The significant decrease in enzyme activity produced by the probiotic and synbiotic combinations of the invention clearly demonstrate a positive effect with regard to the decreasing of the formation of carcinogenic substances.

5.3 Enterolactone content

- 20 **[0072]** Enterolactone content was determined using the method of Adlercreutz et al. (Adlercreutz, H., Fostis, T., Lampe, J., Wähälä, K., Mäkelä, T., Brunow, G. and Hase, T. 1993. Quantitative determination of lignans and isoflavonoids in plasma of omnivorous and vegetarian women by isotope dilution gas-chromatography mass-spectrometry. Scan J. Clin Lab Invest 53: 5 - 18.)

- 25 **[0073]** The enterolactone levels of test persons with an initial enterolactone level of <10 nmol/l increased significantly as a result of the synbiotic intake (to a level of 11.2).

30 **[0074]** No changes were observed during the test in the enterolactone levels of persons whose serum enterolactone level was normal (10<x>30) already at the beginning of the test. The results are shown in Table 9.

Table 9. Enteronelactone contents during the test periods (grouping on the basis of initial level)

	10<x<30 nmol/l	<10 nmol/l
Initial level	24.4	3.2
Probiotic	19.8	2.6
Synbiotic	23.9	11.2

5 **[0075]** Enterolactone content has been shown to clearly correlate with the risk of getting cancer: the higher the content, the lower the risk. This result thus also shows the beneficial effect of the probiotic and synbiotic combinations of the invention to a decreased cancer risk.

5.4. Immunological studies

10 **Effects on lymphocyte function**

[0076] Lymphocyte function was studied before the intake of the synbiotic product was started and after 4 weeks after the intake had been started.

[0077] The lymphocyte function was studied as follows:

15 **[0078]** Lymphocytes were isolated from peripheral blood using Ficoll gradient. The lymphocytes were stimulated with PHA mitogen (Sigma) in RPMI cultivation broth (National Public Health Institute; department of nutrient broths) which contained 5% of inactivated AB+ serum (Finnish Red Cross) and L-glutamine. After 48 hours, cell culture medium was collected for cytokine
20 determination from four adjacent culture wells having a cell density of 200 000 cells per 200 µl of culture broth in the well, either with or without the mitogen. The cells were harvested after 16 hours from the adding of thymidine and the incorporation into DNA (cpm) of radioactive thymidine was measured. The contents of cytokines IL-4, IL-5, TGF-β1 and IFN-γ were determined from the
25 cell culture broths by using the ELISA method.

[0079] During the follow-up, no changes were detected in the IL-4, IL-5 and TGF-β1 contents secreted by the lymphocytes. The IFN-γ content secreted by PHA-stimulated lymphocytes increased significantly during the follow-up (p=0.009, Wilcoxon test, see Figure 1). Both spontaneous and PHA-
30 stimulated proliferation of lymphocytes increased during the follow-up (p=0.0002 in both cases, Wilcoxon test, Figures 1 and 2).

[0080] According to the study results, the use of the synbiotic product thus enhances the proliferation of the lymphocytes and the secretion of IFN- γ cytokine of the test persons. IFN- γ belongs to what are known as Th1 cytokines, which strengthen cytotoxic lymphocyte function and are antagonists of IL-4 and TGF- β 1 cytokines. Low IFN- γ secretion has been reported for persons prone to allergies. In addition, children with a predisposition to atopy and allergy reactions have been presumed to suffer from slow maturing of IFN- γ secretion. The significant stimulating effect of the combinations of the invention on IFN- γ secretion thus proves their efficiency in the prevention and treatment of allergies.

CLAIMS

1. A combination **characterized** in that it comprises at least three of the microorganisms *Lactobacillus rhamnosus* LGG (ATCC 53103), *Lactobacillus rhamnosus* LC705 (DSM 7061), *Propionibacterium freudenreichii* ssp. *shermanii* PJS (DSM7067) and a bifidobacterium, or *Propionibacterium freudenreichii* ssp. *shermanii* PJS (DSM7067) and a bifidobacterium.
2. A combination according to claim 1, **characterized** in that it comprises *Lactobacillus rhamnosus* LGG (ATCC 53103), *Lactobacillus rhamnosus* LC705 (DSM 7061), *Propionibacterium freudenreichii* ssp. *shermanii* PJS (DSM7067) and a bifidobacterium.
3. A combination according to claim 1, **characterized** in that it comprises *Propionibacterium freudenreichii* ssp. *shermanii* PJS (DSM7067) and a bifidobacterium.
4. A combination according to any one of claims 1 to 3, **characterized** in that the bifidobacterium is *Bifidobacterium infantis* Bbi99 (DSM 13692).
5. A combination according to any one of claims 1 to 4, **characterized** in that it further comprises conventional starter microbes.
6. A combination according to any one of claims 1 to 5, **characterized** in that it also comprises a prebiotic or that it is used together with a prebiotic.
7. A combination according to claim 6, **characterized** in that the prebiotic is an oligosaccharide, xylo-oligomer or polydextrose.
8. A combination according to claim 7, **characterized** in that the prebiotic is a galacto-oligosaccharide (GOS).
9. A combination according to any one of claims 1 to 8 to be used as a therapeutic substance.
10. A combination comprising at least two of the following microorganisms: *Lactobacillus rhamnosus* LGG (ATCC 53103), *Lactobacillus rhamnosus* LC705 (DSM 7061), *Propionibacterium freudenreichii* ssp. *shermanii* PJS (DSM7067) and a bifidobacterium, although not the combination *Lactobacillus rhamnosus* LGG (ATCC 53103) and a bifidobacterium, to be used as a therapeutic substance.

23

11. Use of a combination according to any one of claims 1 to 8 in food industry, pharmaceutical industry, or in the manufacture of health-promoting products or natural products.

5 12. Use according to claim 11, **characterized** in that the combination is added to a dairy product, drink, juice, soup or children's food.

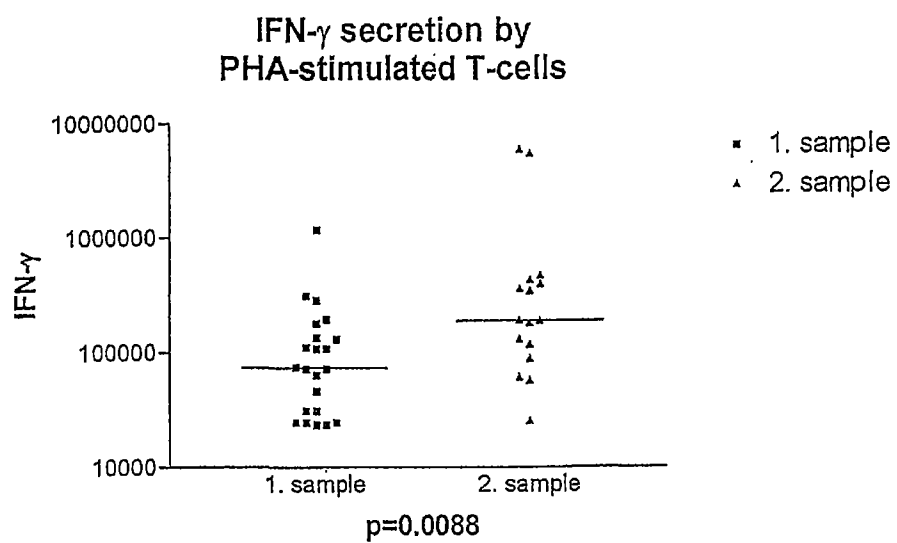
13. Use according to claim 11, **characterized** in that the combination is prepared in a single dose form.

14. Use according to claim 13, **characterized** in that the dose form is a capsule.

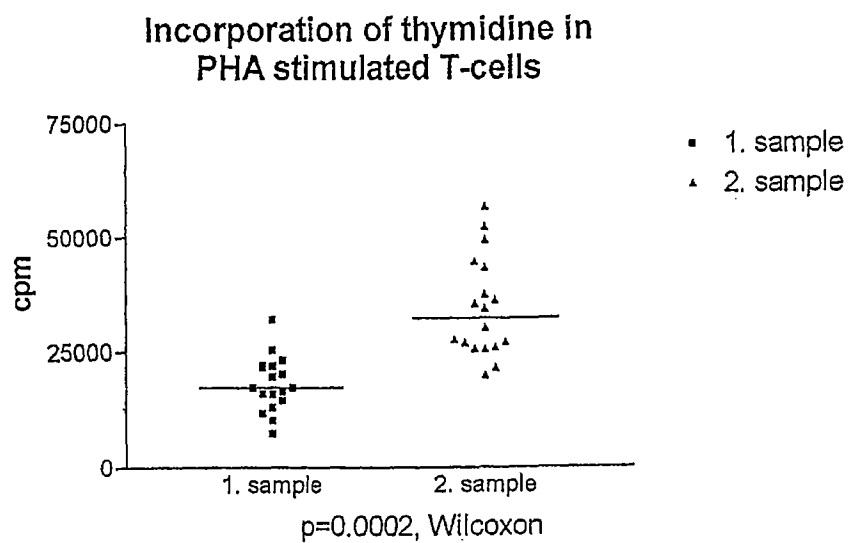
10

1/2

FIGURE 1.




2/2

FIGURE 2.

Valio Ltd.
R & D
P.O. Box 30
00039 Valio
FINLAND

VIABILITY STATEMENT
issued pursuant to Rule 10.2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name: Valio Ltd. R & D Address: P.O. Box 30 00039 Valio FINLAND	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 13692 Date of the deposit or the transfer ¹ : 2000-08-28
III. VIABILITY STATEMENT	
The viability of the microorganism identified under II above was tested on 2000-08-28 ² . On that date, the said microorganism was <input checked="" type="checkbox"/> ³ viable <input type="checkbox"/> ³ no longer viable	
IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED ⁴	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Address: Mascheroder Weg 1b D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):  Date: 2000-08-31

¹ Indicate the date of original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).

² In the cases referred to in Rule 10.2(a) (ii) and (iii), refer to the most recent viability test.

³ Mark with a cross the applicable box.

⁴ Fill in if the information has been requested and if the results of the test were negative.

Valio Ltd.
R & D
P.O. Box 30
00039 Valio
FINLAND

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: Bbi 99	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 13692
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
<p>The microorganism identified under I. above was accompanied by:</p> <p>(X) a scientific description (X) a proposed taxonomic designation</p> <p>(Mark with a cross where applicable).</p>	
III. RECEIPT AND ACCEPTANCE	
<p>This International Depositary Authority accepts the microorganism identified under I. above, which was received by it on 2000-08-28 (Date of the original deposit)¹.</p>	
IV. RECEIPT OF REQUEST FOR CONVERSION	
<p>The microorganism identified under I above was received by this International Depositary Authority on (date of original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion).</p>	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
<p>Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH</p> <p>Address: Mascheroder Weg 1b D-38124 Braunschweig</p>	<p>Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):</p> <p><i>V. Wachs</i></p> <p>Date: 2000-08-31</p>

¹ Where Rule 6.4 (d) applies, such date is the date on which the status of international depositary authority was acquired.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/FI 02/00035

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: A23L 1/03, A61K 35/74

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: A23L, A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Lait, volume 79, 1999, Tarja H. Suomalainen et al: "Propionic acid bacteria as protective cultures in fermented milks and breads", page 165 - page 174, see abstract --	1-14
X	Clinical and Diagnostc Laboratory Immunology, Volume 6, No. 6, November 1999, Pirkka V. Kirjavainen et al: "Effects of Orally Administered Viable Lactobacillus rhamnosus GG and Propionibacterium freudenreichii subsp. shermanii JS on Mouse Lymphocyte Proliferation", page 799 - page 802, see abstract --	1-14

☒ Further documents are listed in the continuation of Box C.☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

29 April 2002

Date of mailing of the international search report

02-05-2002

Name and mailing address of the ISA/
Swedish Patent Office
Box 5055, S-102 42 STOCKHOLM
Facsimile No. +46 8 666 02 86

Authorized officer

Yvonne Siösteen/EÖ
Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.

PCT/FI 02/00035

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Milchwissenschaft, Volume 53, No. 11, 1998, S. Sarkar et al: "Process for the manufacture of a new modified cultured milk product for infants and children", page 603 - page 605 --	1-14
A	US 5378458 A (ANNIKA MÄYRÄ-MÄKINEN ET AL), 3 January 1995 (03.01.95) --	1-14
A	WO 0071138 A2 (REID, GREGOR), 30 November 2000 (30.11.00) --	1-14
A	EP 1020123 A1 (SITIA-YOMO S.P.A.), 19 July 2000 (19.07.00) --	1-14
A	WO 9734615 A1 (THE UNIVERSITY OF NEW SOUTH WALES), 25 Sept 1997 (25.09.97) -- -----	6-8

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/FI 02/00035

Patent document cited in search report			Publication date	Patent family member(s)		Publication date
US	5378458	A	03/01/95	AT	173502 T	15/12/98
				DE	69322131 D,T	20/05/99
				DK	576780 T	02/08/99
				EP	0576780 A,B	05/01/94
				SE	0576780 T3	
				ES	2125278 T	01/03/99
				FI	92498 B,C	15/08/94
				FI	922699 A	11/12/93

WO	0071138	A2	30/11/00	AU	4905300 A	12/12/00

EP	1020123	A1	19/07/00	BR	9906141 A	06/02/01
				IL	132856 D	00/00/00

WO	9734615	A1	25/09/97	AU	705095 B	13/05/99
				AU	2018297 A	10/10/97
				AU	PN881396 D	00/00/00
				CA	2249361 A	25/09/97
				EP	0888118 A	07/01/99
				JP	2000506870 T	06/06/00
				US	6221350 B	24/04/01

100482

Time of Request: Monday, November 06, 2006 04:30:59 EST
Client ID/Project Name: 0110668.00128.1604.mjp
Number of Lines: 188
Job Number: 1841:128013082

Research Information

Service: LEXSEE(R) Feature
Print Request: Current Document: 1
Source: Get by LEXSEE(R)
Search Terms: 1976 U.S. App. LEXIS 8380

Note: Pederson v. Stewart-Warner Corp., 536 F.2d 1179

Send to: PEAL, MARY JANE
BELL BOYD & LLOYD
70 W MADISON ST STE 3100
CHICAGO, IL 60602-4244

LEXSEE

**WALTER H. PEDERSON, resident of Minnesota, Plaintiff-Appellant, v.
STEWART-WARNER CORPORATION, a corporation of the State of Virginia, De-
fendant-Appellee**

No. 75-1915

UNITED STATES COURT OF APPEALS FOR THE SEVENTH CIRCUIT

536 F.2d 1179; 1976 U.S. App. LEXIS 8380; 192 U.S.P.Q. (BNA) 20

February 10, 1976, Argued
June 23, 1976, Decided

PRIOR HISTORY: [**1]

Appeal from the United States District Court for the Northern District of Illinois, Eastern Division - No. 73 C 2607 BERNARD M. DECKER, Judge.

DISPOSITION:

AFFIRMED.

JUDGES:

Fairchild, Chief Judge, Tone and Bauer, Circuit Judges.

OPINION BY:

TONE

OPINION:

[*1179] TONE, Circuit Judge.

After a jury had found plaintiff's patent for a snowmobile speedometer valid and wilfully infringed by defendant, the district judge entered judgment for defendant notwithstanding the verdict on the ground that the patent was invalid for obviousness. 400 F. Supp. 1262 (N.D. Ill. 1975). We affirm.

Plaintiff Walter H. Pederson developed a snowmobile speedometer in preparation for a snowmobile expedition to the North Pole [*1180] to be undertaken in the summer of 1966. The device he developed proved successful on the trip and served as a prototype for speedometers he began manufacturing and selling in 1967. His application for a patent on the device filed later that same year resulted in the issuance in November 1969 of Patent No. 3,478,606, which is before us. Pederson brought this infringement suit based on the patent in

1973 seeking an injunction and damages. Defendant raised [**2] the issues of infringement and invalidity for obviousness. See 35 U.S.C. § 103. We need to reach only the issue of obviousness.

The patent describes a device for transferring the rotational movement of a snowmobile's drive shaft onto a speedometer cable: A conically-shaped adapter or bushing is first inserted into the end of the hollow drive shaft and may be held in place by various means, including friction, threads, projections on the inner wall of the drive shaft, or a spring device arranged inside the shaft. The adapter is then connected to a gear assembly, by means of a short, square shaft, one end of which is fitted into the bushing, and the other into the gear assembly. The gear assembly contains an input gear, which receives the full rotational movement of the drive shaft, an output gear, which rotates proportionately to the input gear, and a transfer device, such as a chain, for transferring the motion from the input gear to the output gear. The purpose of the gear assembly is to vary selectively the rotational movement, depending on the features of the snowmobile and the requirements of the speedometer. The output gear is then attached to a flexible [**3] cable, which carries the motion to the speedometer head, which is usually mounted on the dashboard of the snowmobile. The patent claims cover the drive shaft adapter, the various means of holding it in place, and the gear assembly. For a more complete description see 400 F. Supp. at 1265 n.2.

1.

In arguing that the Patent Act of 1952, 35 U.S.C. § 103, replaced the requirement of "invention," *Hotchkiss v. Greenwood*, 52 U.S. (11 How.) 248, 267, 13 L. Ed. 683 (1851), with that of "non-obviousness," plaintiff overlooks the constitutional basis for the requirement of "invention." See *Sakraida v. Ag Pro, Inc.*, 425 U.S. 273,

280, 96 S. Ct. 1532, 47 L. Ed. 2d 784, 44 U.S.L.W. 4477, 4479 (1976). The *Sakraida* case reaffirms the holding of *Graham v. John Deere Co.*, 383 U.S. 1, 17, 15 L. Ed. 2d 545, 86 S. Ct. 684 (1966), that section 103 did not change the law, and that the standard stated in section 103 is a codification of judicial precedents requiring invention. Thus the requirement of non-obviousness is a paraphrase of the requirement of invention.

2.

Obviousness is a question of law. [**4] *E-T Industries, Inc. v. Whittaker Corp.*, 523 F.2d 636, 641 (7th Cir. 1975); *Panther Pumps & Equipment Co. v. Hydrocraft, Inc.*, 468 F.2d 225, 227 (7th Cir. 1972), cert. denied, 411 U.S. 965, 36 L. Ed. 2d 685, 93 S. Ct. 2143 (1973); *Armour & Co. v. Wilson & Co.*, 274 F.2d 143, 156 (7th Cir. 1960). To decide that question determinations of fact must also be made:

"Under § 103, the scope and content of the prior art are to be determined; differences between the prior art and the claims at issue are to be ascertained; and the level of ordinary skill in the pertinent art resolved." *Graham v. John Deere Co.*, supra, 383 U.S. at 17.

See also *Sakraida v. Ag Pro, Inc.*, supra, 425 U.S. at 280. When, as here, the case is tried to a jury and a general verdict is rendered on the question of validity, disputed factual questions are presumed to have been resolved favorably to the party in whose favor the verdict was returned. *Panther Pumps & Equipment Co. v. Hydrocraft, Inc.*, supra, 468 F.2d at 228. On the basis of the facts so determined, the court must [**5] then decide the issue of obviousness. *Id.* Because obviousness is a question of law, opinions of experts on that question are not among the facts presumptively decided in the winning party's favor, and the District Court in this case was therefore not bound to accept expert testimony offered by plaintiff as to what [*1181] would not have been obvious to one with ordinary skill in the art.

The District Court's opinion invalidating the patent for obviousness discusses at some length evidence presented at trial on the scope and content of the prior art and the differences between the prior art and the Pederson patent. See 400 F. Supp. at 1264-1268. Plaintiff argues that the district judge erred in failing to make a finding as to the level of ordinary skill in the art as required by *Graham*, supra, 383 U.S. at 17. This argument overlooks the fact that, because plaintiff chose to demand

a jury, the trier of facts was the jury, not the court. If plaintiff wanted specific findings on that issue he should have asked the court to submit an appropriate special verdict under *Rule 49(a)*, *Fed. R. Civ. P.*, although whether to submit such a verdict was for [**6] the trial court's discretion. There was, we note, uncontested evidence showing that the design and manufacture of instruments that measure the speed of rotation is an old art in which defendant has long been engaged.

3.

The Supreme Court has recently reminded us, in reversing a decision of the Fifth Circuit which had upheld a patent claiming a new combination of old elements that combination claims should be "[scrutinized] . . . with a care proportioned to the difficulty and improbability of finding invention in an assembly of old elements." *Sakraida v. Ag Pro, Inc.*, supra, 425 U.S. at 281, quoting from *Great Atlantic & Pacific Tea Co. v. Supermarket Equipment Corp.*, 340 U.S. 147, 152, 95 L. Ed. 162, 71 S. Ct. 127 (1950). This court has often recognized that such a claim must pass a "rather severe test" of obviousness. *Gettelman Mfg. Inc. v. Lawn 'N' Sport Power Mower Sales & Service, Inc.*, 517 F.2d 1194, 1197 (7th Cir. 1975); *Panduit Corp. v. Burndy Corp.*, 517 F.2d 535, 539 (7th Cir. 1975), cert. denied, 423 U.S. 987, 96 S. Ct. 395, 46 L. Ed. 2d 304 (1975); *Skil Corp. v. Lucerne Products, Inc.*, 503 F.2d 745, 749 (7th Cir. 1974); [**7] cert. denied, 420 U.S. 974, 43 L. Ed. 2d 654, 95 S. Ct. 1398 (1975); *Toro Manufacturing Corp. v. Jacobsen Manufacturing Co.*, 357 F.2d 901, 904 (7th Cir. 1966). That test, stated "about as precisely as the subject permits," was set forth in *Great Atlantic & Pacific Tea Co. v. Supermarket Equipment Corp.*, supra, 340 U.S. at 151 (1950), quoting *Lincoln Engineering Co. v. Stewart-Warner Corp.*, 303 U.S. 545, 549, 82 L. Ed. 1008, 58 S. Ct. 662 (1938): "The mere aggregation of a number of old parts or elements which, in the aggregation, perform or produce no new or different function or operation than that theretofore performed or produced by them, is not patentable invention." The Court added: "The conjunction or concert of known elements must contribute something; only when the whole in some way exceeds the sum of its parts is the accumulation of old devices patentable." 340 U.S. at 152. See also *Anderson's-Black Rock, Inc. v. Pavement Salvage Co.*, 396 U.S. 57, 61, 24 L. Ed. 2d 258, 90 S. Ct. 305 (1969). The Court applied these principles in *Sakraida*, holding that a patent which [**8] "simply arranges old elements with each performing the same function it had been known to perform, although perhaps producing a more striking result than in previous combinations" is "not patentable under standards appropriate for a combination patent." 425 U.S. at 282.

4.

At trial defendant introduced several prior art references into evidence. The first was a snowmobile speedometer that functioned for some 700 miles on a cross-country trip through Alaska in 1960. That speedometer was driven by inserting one end of the cable into the end of a rotating shaft which in turn was driven by the main drive shaft. Plaintiff attacks this evidence, which is largely based on oral testimony, on various grounds and argues that the drive arrangement proved unsuccessful on the trip and was abandoned thereafter. It is questionable whether such a limited and unpublicized use would qualify as "prior art," cf. *Deep Welding, Inc. v. Sciaky Bros., Inc.*, 417 F.2d 1227, 1235 (7th Cir. 1969), [*1182] cert. denied, 397 U.S. 1037, 25 L. Ed. 2d 648, 90 S. Ct. 1354 (1970); *Deller's Walker on Patents* § 107 at 114, 124 (2d ed. 1964); the evidence [**9] would, however, be relevant to show the ordinary skill in the art. We cannot tell, of course, how the jury evaluated this evidence, but we need not rely upon it to determine the question of obviousness, for there is other stronger evidence of analogous applications of the elements of the claimed invention.

In 1959 and 1960 International Harvester Company built, and sold to the New York City Sanitation Department, over 100 tractors having a speedometer-drive arrangement which was similar in important respects to the claimed combination. The components were furnished by defendant. In this arrangement the speedometer cable was driven by attaching one end of it to a "right angle gear assembly." The gear assembly was engaged and driven by a "drive tip," or short metal shaft, which had a bushing attached to its opposite end. The drive tip and bushing were then pressed into the hollow end of a brake shaft, which drove one of the tractor's rear wheels.

Also pertinent is evidence relating to tachometers, which measure engine revolutions rather than wheel revolutions and are applied by means of a flexible cable to an engine take-off instead of a take-off proportioned to road speed. Defendant's [**10] 1966 instrument catalogue shows, among various kinds of tachometer-drive arrangements offered by defendant for sale, one which utilized a hole drilled in the end of a rotating shaft, into which was inserted a bushing and the flexible cable connected to the tachometer head. Another drive shown in the catalogue also took its rotational input from the hollowed end of a rotating shaft.

The tractor speedometers and defendant's tachometers were applications to which one with skill in the art could "reasonably be expected to look for solutions to the problems which the patented device attempts to solve," *Fischer & Porter Co. v. Haskett*, 354 F. Supp. 464, 477 (E.D. Pa. 1973), regardless of the inventor's actual awareness of those applications, *Arrow Safety Device Co. v. Nassau Fastening Co.*, 496 F.2d 644, 646

(3d Cir. 1974). These prior-art devices establish that measuring the speed of a rotating shaft by a means connected to the inner wall of one end of the shaft was known well before plaintiff's alleged invention. Plaintiff's combination of elements did not create a new technique for making such a measurement. Application of the take-off apparatus [**11] to the drive shaft rather than a brake shaft, as in the tractor speedometers, is not a patentable distinction. Plaintiff himself testified that the take-off could be made from any shaft rotating in proportion with the drive shaft.

Plaintiff seeks to distinguish the tractor speedometers because, being attached to a shaft which drove the left wheel, they registered inaccurately on left turns. This fact, however, does not make their teaching irrelevant. "Under § 103 . . . a claimed invention may be obvious, even though it is not identically disclosed or described by the prior art; it is sufficient that the subject matter of the patented article, taken as a whole, has been disclosed by the prior art." *Panduit Corp. v. Burndy Corp.*, supra, 517 F.2d at 538-539. For the same reason, connecting the flexible cable to a speedometer head, rather than a tachometer head, and placing the drive components in a snowmobile, rather than some other vehicle, are not patentable ideas. Compare *Research Corp. v. Nasco Industries, Inc.*, 501 F.2d 358, 360 (7th Cir. 1974), cert. denied, 419 U.S. 1096, 42 L. Ed. 2d 688, 95 S. Ct. 689 (1974). See also [**12] *Ling-Temco-Vought, Inc. v. Kollsman Instrument Corp.*, 372 F.2d 263, 268 n.1 (2d Cir. 1967); *Skee-Trainer, Inc. v. Garelick Mfg. Co.*, 361 F.2d 895, 898 (8th Cir. 1966). "Whether treated as prior art or as evidence of the state of skill in the art," *Skil Corp. v. Lucerne Products, Inc.*, supra, 503 F.2d at 749, the undisputed reference relied upon by defendant show that "this particular use of the assembly of old elements would be obvious to any person skilled in the art of mechanical application," *Sakraida v. Ag Pro, Inc.*, supra, 425 U.S. at 282. *

* As we said earlier, because obviousness is a question of law and reviewable as such, we are not bound by the jury's acceptance of the testimony of Pederson's expert, who gave the opinion that the patent was not obvious. It is not clear, moreover, that his opinion took into consideration the prior art cited by defendant. He did examine the five patents cited against the Pederson application by the Patent Office, which neither party offered in evidence. Defendant's expert, however, gave a negative response when asked whether "any of the five patents . . . cited by the Patent Office show speedometer installations in which a bushing is positioned in a hole in the end of a rotating shaft."

536 F.2d 1179, *, 1976 U.S. App. LEXIS 8380, **;
192 U.S.P.Q. (BNA) 20

[**13]

[*1183] With respect to the patent's commercial success and defendant's failure to develop a snowmobile speedometer earlier than it did, we have nothing to add to the District Court's excellent analysis. See *400 F. Supp.*

at 1269-1270. Both factors are relevant to the question of patentability, but, as the District Court said, "they cannot, by themselves, give rise to a finding or conclusion of non-obviousness." See also *Sakraida v. Ag Pro, Inc.*, *supra*, 425 U.S. 273, 44 U.S.L.W. at 4480.

AFFIRMED.

100482

***** Print Completed *****

Time of Request: Monday, November 06, 2006 04:30:59 EST

Print Number: 1841:128013082

Number of Lines: 188

Number of Pages:

Send To: PEAL, MARY JANE
BELL BOYD & LLOYD
70 W MADISON ST STE 3100
CHICAGO, IL 60602-4244